

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Hans-Ulrich Demuth, Torsten Hoffman, Dagmar Schlenzig
and Ulrich Heiser

Attorney Docket No: 20784/6

Title: *New Effectors of Dipeptidyl Peptidase IV for Topical Use*

CERTIFICATE OF EXPRESS MAIL

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the United States Postal Service on the date shown below with sufficient postage as express mail number ET 720934774 US in an envelope addressed to the: Box Patent Application, Assistant Commissioner for Patents, Washington, D.C. 20231 on:

By:

Deborah Celeste

Date

February 22, 2002

10032001 03:00



5 *NEW EFFECTORS OF DIPEPTIDYL PEPTIDASE IV FOR TOPICAL USE*

RELATED APPLICATIONS

This application claims the benefit of International Patent Application No.
PCT/EP00/08118, filed 20 August 2000, published 01 March 2001 as
10 WO 01/14318, which claims priority to German Patent Application No. DE 199 40
130.6, filed 24 August, 1999.

BACKGROUND OF THE INVENTION

15 *Field of the Invention.*

The invention relates to new effectors of dipeptidyl peptidase IV (DP IV) and
methods of treatment comprising the topical application thereof. These effectors
can be used for targeted influencing of locally limited pathophysiological and
physiological processes (inflammation, chemotaxis, autoimmune diseases wound
20 healing), wherein the enzymatic activity and binding activities of dipeptidyl
peptidase IV and of enzymes having comparable or identical activity and of
proteins having a related primary structure (e.g. FAP, Fibroblast Activation Protein
(Levy *et al.*, 1999)) are influenced by means of effectors (substrates, pseudo-
substrates, inhibitors, antibodies, binding proteins, binding antagonists, binding
25 agonists, *inter alia*).

Related Art.

In addition to proteases involved in non-specific proteolysis, which results, finally,
in the breakdown of proteins into amino acids, regulatory proteases are known,
30 which take part in the functionalization (activation, deactivation, modification) of
endogenous peptide active substances (Kirschke *et al.*, 1995; Kräusslich and
Wimmer, 1987). Especially in the context of immunological research and
neuropeptide research, a number of such so-called convertases, signal peptidases or
enkephalinases have been discovered (Gomez *et al.*, 1988; Ansorge *et al.*, 1991).
35 Because of the frequency of the presence of the amino acid proline in a multiplicity

5 of peptide hormones and because of the related structural properties of those peptides, a function analogous to the signal peptidases is being discussed for proline-specific peptidases (Yaron and Naider, 1993; Vanhoof *et al.* 1995). As a result of its particular structure, proline in those peptides determines both the conformation and stability of those peptides, protecting them from breakdown by
 10 non-specific proteases (Kessler, 1982). Enzymes that, in contrast, act in highly specific, structure-modifying manner on proline-containing sequences (HIV-protease, cyclophilin, *inter alia*) are attractive targets for current active substance research. In particular, for the peptidases prolyl endopeptidase (PEP) and dipeptidyl peptidase IV (DP IV), which cleave after the proline, it has been possible
 15 to conclude that there probably are connections between modification of the biological activity of natural peptide substrates and selective cleavage thereof by those enzymes. It is accordingly postulated that PEP plays a part in learning and in the memory process and that DP IV is involved in signal transmission during the immune response (Ishiura *et al.*, 1989; Hegen *et al.*, 1990).
 20 DP IV activity and DP IV-analogous activity (for example, the lysosomal DP II has a substrate specificity that is almost identical to DP IV) is to be found in the bloodstream and in almost all organs, where it cleaves dipeptides from the N terminus of biologically active peptides with high specificity when their sequence contains proline or alanine as residues adjacent to the N-terminal amino acid. It is
 25 therefore assumed that this enzyme is involved in regulating the biological activity of polypeptides *in vivo* (Vanhoof *et al.*, 1995).

It has recently been shown that a series of chemokines (RANTES, SDF-1 alpha, MDC, eotaxin, *inter alia*) are substrates of DP IV and that they are modulated in their function by DP IV (Proost *et al.*, 1998; Proost *et al.*, 1998; Proost *et al.*, 1999;
 30 Shioda *et al.*, 1998). As a result of their chemotactic action, chemokines are substantially involved in the regulation of local immunological processes, such as autoimmune processes, inflammation and wound healing (Nelson and Krensky, 1998). In more recent work, we have been able to demonstrate that biologically active peptides having serine or threonine in the P₁-position (glucagon, VIP,
 35 PACAP) are also substrates of DP IV.
 A series of biologically active DP IV-substrates (substance P, somatostatin, VIP,

5 PACAP, *inter alia*) are involved in the regulation of neuronal, immunological and vasoactive processes in the skin (Scholzen *et al.*, 1998); (Wallengren, 1997). Dipeptidyl peptidase IV accordingly represents an important control centre in regulating the activity of gastrointestinally, immunologically and neurologically active peptides and, consequently, is an interesting therapeutic target (Augustyns *et*
10 *al.*, 1999). The precise details of the signal cascades have not, however, been clarified fully.

The role of DP IV in the regulation of blood sugar is known in greater detail. As a result of limited proteolysis, the incretins GIP₁₋₄ and GLP-1₇₋₃₇ are inactivated. Inhibition of plasma-DP IV activity leads, by way of prolonged activity of the
15 incretins and increased insulin release, to normalization of the blood sugar level (Demuth *et al.*, 1996; Pauly *et al.*, 1996; Pauly *et al.*, 1999).

The role of DP IV in the immune system has not yet been fully clarified. It is an activation marker of T-lymphocytes and a receptor for adenosinedeaminase. The use of DP IV-inhibitors has immunosuppressant effects in cell culture and *in vivo*
20 (Ansorge *et al.*, 1995; Reinhold *et al.*, 1997; Kubota *et al.*, 1992). Using monoclonal antibodies against CD26, stimulatory effects on intracellular signal cascades (Ca²⁺ influx, kinase activations) have been obtained, in some cases independently of the enzymatic activity of the enzyme (Hegen *et al.*, 1993; Kameoka *et al.*, 1995; Tanaka *et al.*, 1993; Kähne *et al.*, 1995).

25 Lysyl-prolyl analogues derived from the N-terminal sequence of substance P have shown a wound-healing-promoting effect, which is attributed to the structural similarity to substance P. In contrast, irreversible DP IV-inhibitors used systemically have resulted in inhibition of wound healing (Buntroek *et al.*, 1988; Kohl *et al.*, 1991; Kohl *et al.*, 1989).

30 In addition to the use of DP IV-inhibitors for the normalization of blood glucose, DP IV-inhibitors have hitherto been used systemically for treating arthritis in an animal model.

In arthritis patients and in animal arthritis models, a reduction in DP IV activity has been observed (Küllertz and Boigk, 1986; Fujita *et al.*, 1992). In particular, as a
35 result of oral or subcutaneous administration of systemically acting DP IV-inhibitors, suppression of alkyldiamine-induced arthritis has been achieved in an

5 animal model (Tanaka *et al.*, 1997; Tanaka *et al.*, 1998).

In relation to other autoimmune diseases as well, an effect has been obtained using DP IV-inhibitors. For example, as a result of DP IV inhibition it has been possible to achieve suppression of the proliferation of myelin basic protein-specific T cell clones (Reinhold *et al.*, 1998).

10 In the case of various skin diseases (psoriasis, lichen planus) and cancerigenic diseases of the skin, it has been possible to demonstrate increased DP IV activity in keratinocytes and fibroblasts (Novelli *et al.*, 1996; Raynaud *et al.*, 1992).

Fibroblast activation protein, which is closely related to DP IV, having approximately 50% sequence homology with respect to DP IV, and which is

15 probably the same as the seprase described by Piñieiro-Sanchez *et al.*, 1997, is also expressed to an increased extent by inactivated fibroblasts of epithelial carcinomas and healing wounds (Niedermeyer *et al.*, 1998).

Because of the wide distribution of the protein in the body and the wide variety of mechanisms involving DP IV, DP IV activities and DP IV-related proteins,

20 systemic therapy (enteral or parenteral administration) with DP IV-inhibitors can result in a series of undesirable side-effects. For example, parenteral or enteral administration of DP IV-inhibitors will intervene in a regulating or deregulating manner in glucose metabolism.

It has now been possible to show that side chain-modified substrates of the enzyme

25 dipeptidyl peptidase IV can be recognized by the enzyme and cleaved in the same way as unmodified substrates (Demuth, H.-U., Heins, J., 1995).

For example, it has been possible to show that phosphorylated dipeptide-(B)-*p*-nitroanilides (KASPARI, A., *et al.*, 1996) are substrates of DP IV. DP IV-inhibitors such as, for example, Glu(Gly)-Thia or Lys(Z-NO₂)-Thia (Reinhold, D.,

30 *et al.*, 1998) are transported completely.

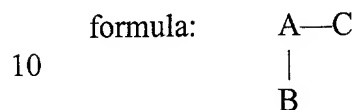
BRIEF SUMMARY OF THE INVENTION

The problem to be solved consisted in preparing compounds that can be used for targeted influencing of locally limited pathophysiological and physiological

35 processes. The problem of the invention especially consists in obtaining locally limited inhibition of DP IV or DP IV-analogous activity for the purpose of targeted

5 intervention in the regulation of the activity of locally active peptide hormones.

The problem is solved according to the invention by providing compounds of the general



Wherein

15 A is an amino acid having at least one functional group in the side chain,

B is a chemical compound covalently bound to at least one functional group of the side chain of A, chosen from the group consisting of:

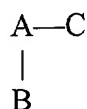
- oligopeptides having a chain length of up to 20 amino acids, excluding homopolymers of glycine consisting of up to 6 glycine monomers, and

20 - polyethylene glycols having molar masses of up to 20,000 g/mol, and

C is a thiazolidine, pyrrolidine, cyanopyrrolidine, hydroxyproline, dehydroproline or piperidine group amide-bonded to A.

In accordance with the invention, at least one pharmaceutical composition is especially provided which comprises

25 at least one compound of the general formula:



wherein

30 A is an amino acid, preferably an α -amino acid, especially a natural α -amino acid having at least one functional group in the side chain, preferably threonine, tyrosine, serine, arginine, lysine, aspartic acid, glutamic acid or cysteine,

B is a chemical compound covalently bound to at least one functional group in the side chain of A, namely oligopeptides having a chain length of up to 20 amino

35 acids, polyethylene glycols having molar masses of up to 20 000 g/mol, optionally substituted organic amines, amides, alcohols, acids or aromatic compounds having from 8 to 50 C atoms,

C is a thiazolidine, pyrrolidine, cyanopyrrolidine, hydroxyproline, dehydroproline or piperidine group amide-bonded to A,

40

5 and

at least one customary adjuvant appropriate for the site of action.

Furthermore, such compounds or pharmaceutical compositions are used for influencing topically especially reducing the activity of dipeptidyl peptidase IV or analogous enzymes. Such compounds or pharmaceutical compositions may be used in the form of an ointment, cream, cosmetic, patch, dressing, drops, spray, inhalation, implant or injection solution.

BRIEF DESCRIPTION OF THE DRAWING

15 *Figure 1: Systemic action of orally administered DP IV-inhibitors in healthy Wistar rats.*

The course, over time, of the percentage inhibition of plasma-DP IV activity following oral administration of 5 μ mol of inhibitor per 300 g of rat (n = 2) is shown.

DETAILED DESCRIPTION OF THE INVENTION

Throughout the description and the claims, the expression "alkyl" can denote a C₁₋₅₀alkyl group, preferably a C₁₋₃₀alkyl group, especially a C₈₋₁₂alkyl group; for example, an alkyl group may be a methyl, ethyl, propyl, isopropyl or butyl group.

25 The expression "alk", for example in the expression "alkoxy", and the expression "alkan", for example in the expression "alkanoyl", are defined as for "alkyl".

Aromatic compounds are preferably substituted or optionally unsubstituted phenyl, benzyl, naphthyl, biphenyl or anthracene groups, which preferably have at least 8 C atoms.

30 The expression "alkenyl" can denote a C₂₋₁₀alkenyl group, preferably a C₂₋₆alkenyl group, which has the double bond(s) at any desired location and may be substituted or unsubstituted.

The expression "alkynyl" can denote a C₂₋₁₀alkynyl group, preferably a C₂₋₆alkynyl group, which has the triple bond(s) at any desired location and may be substituted or unsubstituted.

35 The expression "substituted" or substituent can denote any desired substitution by

- 5 one or more, preferably one or two, alkyl, alkenyl, alkynyl, mono- or multi-valent acyl, alkanoyl, alkoxyalkanoyl or alkoxyalkyl groups; the afore-mentioned substituents may in turn have one or more (but preferably zero) alkyl, alkenyl, alkynyl, mono- or multi-valent acyl, alkanoyl, alkoxyalkanoyl or alkoxyalkyl groups as side groups.
- 10 Organic amines, amides, alcohols or acids, each having from 8 to 50 C atoms, preferably from 10 to 20 C atoms, can have the formulae (alkyl)₂N- or alkyl-NH-, -CO-N(alkyl)₂ or -CO-NH(alkyl), -alkyl-OH or -alkyl-COOH.
- Despite an extended side chain function, the compounds according to the invention can still bind to the active centre of the enzyme dipeptidyl peptidase IV and
- 15 analogous enzymes but are no longer actively transported by the peptide transporter PepT1. The resulting reduced or greatly restricted transportability of the compounds according to the invention leads, in ideal manner, to local, topical inhibition of DP IV and of analogous enzymes.
- The compounds according to the invention or compounds used in accordance with
- 20 the invention can be present or used, respectively, in the form of racemates or in the form of enantiomerically pure compounds, preferably in the L-threo or L-allo form with respect to A.
- Local intervention in the regulation of peptide hormones by means of topical administration of specific DP IV-inhibitors accordingly makes it possible to avoid
- 25 systemic side-effects caused by enteral or parenteral administration of DP IV-inhibitors, because only locally limited inhibition of DP IV activity takes place.
- Systemic regulation processes or regulation processes in other tissues remain unaffected to a very considerable extent because rapid systemic distribution of the compounds is avoided.
- 30 By extending/expanding the side chain modifications, for example beyond a number of seven carbon atoms, it is accordingly possible in accordance with the invention to obtain a dramatic reduction in transportability (Table 1). The Examples in Table 1 clearly show that, with increasing spatial size of the side chains, there is a reduction in the transportability of the substances. By spatially
- 35 and sterically expanding the side chains, for example beyond the atom group size of a monosubstituted phenyl radical, hydroxylamine radical or amino acid residue,

5 it is possible according to the invention to modify or suppress the transportability of the target substances.

It is accordingly possible to influence DP IV activity in the living body in discriminating manner.

By means of the invention, it is accordingly possible, on the one hand, to achieve
 10 effective action of the inhibitors in the tissue to be treated and, on the other hand, by virtue of locally limited, that is to say topical, administration of DP IV-inhibitors it is possible to avoid systemic actions of the inhibitors to a very considerable extent. It is accordingly possible to influence local physiological and pathophysiological processes (inflammation, psoriasis, arthritis, autoimmune
 15 diseases, allergies) effectively and with few side-effects.

The invention is supported by the following facts:

DP IV-inhibitors administered enterally and parenterally, that is to say orally and intravenously or subcutaneously, are distributed systemically and inhibit DP IV and analogous activities throughout the body.

20 A series of bioactive peptide substrates of DP IV are, however, involved in the regulation of local signal cascades (chemotaxis, inflammation, neurotransmission).

The side chain-modified DP IV-inhibitors according to the invention exhibit, surprisingly, high inhibitory potency, but are absorbed and
 25 transported hardly at all or not at all and consequently do not result in demonstrable systemic effects.

The invention therefore makes available new DP IV-inhibitors and a novel approach for the use of DP IV-inhibitors *in vivo*. Such inhibitors can be matched to the type of use by means of chemical modifications and/or formulations. For
 30 example, systemic distribution is made difficult or prevented by means of voluminous hydrophilic substitutions on the side chain.

The inhibitors can be administered in pharmaceutical and cosmetic preparations. Topical use encompasses local use of the inhibitors by direct application to the tissue to be treated (e.g. skin, wounds, tumours) by means of ointments, creams or
 35 cosmetics, and indirect application by means of effector-containing patches, dressings or the like, by application in parts of the body (mouth, nose, ears, eyes,

5 lungs) in the form of drops, sprays, inhalations or the like, by direct injection into
or around the tissue to be treated and by implantation of effector-containing
materials. Topical use further encompasses oral or anal administration of non-
absorbable or not readily absorbable effectors of dipeptidyl peptidase IV or of DP
10 DP IV.

In accordance with the invention, there are especially used compounds wherein the
oligopeptides have chain lengths of from 3 to 15, especially from 4 to 10, amino
acids, and/or the polyethylene glycols have molar masses of at least 250 g/mol,
preferably of at least 1500 g/mol and up to 15,000 g/mol, and/or the optionally
15 substituted organic amines, amides, alcohols, acids or aromatic compounds have at
least 12 C atoms and preferably up to 30 C atoms. Furthermore, there are disclosed
pharmaceutical and cosmetic compositions that comprise at least one compound
according to the invention, optionally in combination with carriers or adjuvants
customary *per se*.

20 The compounds or pharmaceutical or cosmetic compositions according to the
invention can be used for topically influencing the activity of dipeptidyl peptidase
IV or of analogous enzymes, especially for the prophylaxis or therapy of diseases
of the skin or mucosa, autoimmune diseases and inflammation such as, for
example, psoriasis, allergies, arthritis, tumours or autoimmune diseases.

25 The compounds and pharmaceutical or cosmetic compositions can be formulated
and used in the form of an ointment, cream, cosmetic, patch, dressing, drops, spray,
inhalation, implant or injection solution.

The adjuvants used in accordance with the invention are known *per se*.

The invention accordingly relates to the topical use of effectors of dipeptidyl
30 peptidase IV and of DP IV-analogous enzyme activities and of DP IV-like proteins.
Topical use allows local modification of the activities of the afore-mentioned
highly specific enzymes which are crucially involved in inactivation and activation
of biologically active peptides (chemokines, substance P, VIP, PHM, PACAP,
growth factors, *inter alia*).

35 Targeted intervention in local immunological processes (chemotaxis, inflammatory
processes, autoimmune diseases) is accordingly possible, as well as effective and

5 targeted treatment of pathophysiological and physiological processes related thereto
 (psoriasis, periodontitis, arthritis, allergies, inflammation). The invention makes it
 possible for the inhibitors to be used simply and in high local concentrations.
 As a result of low systemic loading with the corresponding effectors, an influence
 on the incretin system or systemic immune response is avoided.

10

Examples.

Example 1: Action of side chain-modified glutamylthiazolidines as non-
 readily-transportable DP IV-inhibitors.

15 Side chain-modified glutamylthiazolidines having a structure H-Glu(X)-Thia were
 synthesized, with polyethylene glycol or glycine oligomers of various chain lengths
 being used as X (see Method A for description of synthesis). The binding
 characteristics of those derivatives and their transportability by the peptide
 transporter PepT1 were investigated and the K_i values with respect to DP IV were
 determined (Table 1).

20

 It was found, surprisingly, that the side chain modifications modify the binding
 characteristics to the compound only to a slight extent. In contrast, the ability of
 the inhibitors to be transported by the peptide transporter is dramatically
 diminished by the side chain modification.

25 The said DP IV-inhibitors are therefore excellently suited to achieving locally
 limited (topical) inhibition of DP IV in the body.

5 **Table 1: Transportability and inhibitor constants of selected DP IV-inhibitors.**

Compound	EC ₅₀ (mM) ¹	I _{max} (HA) ²	K _i (mol/l) ³
<i>amino acid thiazolidides</i>			
H-Ile-Thia	0.98	25 ± 8	1.3e-7 ± 11.1 %
H-Glu-Thia	1.1	35 ± 13	6.1e-7 ± 11.4 %
<i>side chain-modified glutamylthiazolidines</i>			
H-Gly(NHOH)-Thia	3.18	42 ± 11	1.7e-6 ± 8.6 %
H-Glu(Gly ₃)-Thia	8.54	n.d. ⁴	1.92e-7 ± 8.4 %
H-Glu(Gly ₅)-Thia	> 10	n.d. ⁴	9.93e-8 ± 11.4 %
H-Glu(PEG)-Thia	> 10	n.d. ⁴	3.11e-6 ± 9.8 %

¹ Effective concentrations of the compounds inhibiting the binding of ³H-D-Phe-Ala (80mM) to PepTl-expressing *P. pastoris* cells by 50 % (EC₅₀ values).

10 ² Transport characteristics at PepTl-expressing oocytes of *X. laevis* - by means of two-electrode voltage clamp method, I = inward currents generated by the transport.

³ Inhibitor constants for competitive inhibition of purified kidney-DP IV by the compounds of the examples.

⁴ Not detectable.

15 Example 2: Effect of orally administered DP IV-inhibitors on activity of serum-DP IV.

The inhibition of plasma-DP IV (systemic action) was investigated following oral administration of side chain-modified DP IV-inhibitors (5μM/300 mg rat) compared with unmodified inhibitors in healthy Wistar rats.

20 Although the inhibitors have approximately equal K_i values to DP IV (Table 1), plasma-DP IV is inhibited by the novel side chain-modified inhibitors much more slowly and to a much lesser extent overall. This means that the inhibitors are absorbed from the intestine much less readily or not at all. In the case of Glu(Gly)₅-Thia, especially, no systemic action of the orally administered active
25 ingredient is detectable.

Those inhibitors may consequently act as basic structures for the synthesis of novel topically administrable DP IV-inhibitors without systemic action.

Example 3: Synthesis of side chain-modified inhibitors of DP IV.

30 **3.1 Synthesis of Boc-Glu-Thia.**

Reaction of Boc-Glu(OMe)-OH with Thia*HCl according to Method B (see section 3.4 for methods), hydrolysis of Boc-Glu(OMe)-Thia according to Method G.

5 **Table 2: Analytical data for Boc-Glu-Thia.**

Compound	Empirical formula M_r Synthesis method Yield	MS $[M+H]^+$ TLC: R_f /system m.p.	$[\alpha]^{20}_D$ Concentration Solvent	Elemental analysis (calc./ found) %	HPLC R_t [min]/system
Boc-Glu- Thia	C ₁₃ H ₂₂ N ₂ O ₅ S 318.38 B+G 62 %	319.5 0.52 / A ¹ 0.42 / B ¹ 115-118°C	-3.1 C=1 methanol	C:49.04/48.89 H:6.96/6.82 N:8.80/8.59	13.93 / A ²

¹ Thin-layer chromatography

System A: chloroform/methanol 90:10

System B: benzene/acetone/acetic acid 25:10:0.5

System C: n-butanol/EA/acetic acid/H₂O 1:1:1:1

10 ² HPLC separation conditions:

Column: Nucleosil C-18, 7 μ , 250 mm x 21 mm; eluant: isocratic, 40 % ACN/water/0.1% TFA;

flow rate: 6 ml/min; λ . = 220 nm.

3.2 Side chain-modified Boc-glutamylthiazolidines.

15 Boc-Glu-Thia was modified at the γ -carboxylic acid function by introducing radicals of varying size. The radicals were coupled by way of their amino group by forming an amide bond to the γ -carboxylic acid function, with a variety of coupling methods being used depending on the radical.

The following amino components were attached to Boc-Glu-Thia using the method
20 stated:

Amino component	Coupling methods (see §3.4)	Yields
Polyethylene glycol amine ($M_r \approx 8000$)	C	93 %
H-Gly-Gly -Gly-OH	D+E	49 %
H-Gly-Gly-Gly-Gly-Gly-OH	D+E	86 %

In 2 cases, purification of the reaction products differs from the general description of synthesis:

25 1. *Boc-Glu(Gly₅)-Thia*: The product already precipitates out from the mixture on stirring overnight; it is subsequently filtered off and washed with 0.1N HCl and copious amounts of water. It is then dried over P₄O₁₀ *in vacuo*.

2. *Boc-Glu(PEG)-Thia*: In contrast to the general procedure, the starting materials for the synthesis are dissolved in a 500-fold excess of DMF. After the reaction is complete, the DMF is completely removed *in vacuo* and the residue is dissolved in
30 a large amount of methanol. After ether is poured on, to form an upper layer, the

- 5 product precipitates out together with the unreacted PEG. Fine purification was carried out by preparative HPLC separation on a gel filtration column (Pharmacia, Sephadex G-25, 90 μ m, 260 mm - 100 mm; Separating conditions: eluant: water; flow rate: 5 ml/min; λ = 220 nm.)

Table 3: Synthesis data for side chain-modified Boc-glutamylthiazolidines.

Compound	Empirical Formula M_r Yield	MS[M+H] ⁺ TLC/R _f / System m.p.	$[\alpha]^{20}_D$ Concentration Solvent	Elemental analysis calc./ found) %	HPLC R _t [min]/system
Boc-Glu(Gly ₃)-Thia	C ₁₉ H ₃₁ N ₅ O ₈ S 489.54 49 %	490.5		C:46.62 H:6.38 N:14.31	
Boc-Glu(Gly ₅)-Thia	C ₂₃ H ₃₇ N ₇ O ₁₀ S 603.64 86 %	604.5 0.09/C decomp. from 202 °C	n.d. ¹	C:45.76/45.60 H:6.18/6.11 N:16.24/16.56	11.93 / A ²
Boc-Glu(PEG)-Thia		≈8000 mass emphasis) 52-53 °C	n.d. ¹	n.d. ¹	n.d. ¹

10 ¹ Not determined, or not determinable.

² HPLC separation conditions

Column: Nucleosil C-18, 7 μ , 250 mm x 21 mm

Eluant: isocratic, 40 % ACN/water/0.1% TFA; Flow rate: 6 ml/min; λ = 220 nm.

15 3.3 Side chain-modified glutamylthiazolidines.

The N-terminal Boc protecting groups were cleaved off the compounds described in Table 3 using method F. The substances modified with Gly derivatives were purified by preparative HPLC separation and are present as trifluoroacetates. The H-Glu(PEG)-Thia was purified on a gel filtration column in the same manner as the

20 Boc-protected precursor.

5

Table 4: Synthesis data for side chain-modified glutamylthiazolidines.

Compound	Empirical Formula M _r Yield	MS [M+H] ⁺ TLC/R _f / System m.p.	[α] ²⁰ _D Concentration Solvent	Elemental analysis (calc./ found) %	HPLC R _t [min]/ system
H-Glu(Gly ₃) Thia *TFA	C ₁₆ H ₂₄ N ₅ O ₈ SF ₃ 503.45 94 %	503.45 0.32 / C 91-94°C	= 4.1 c = 1 methanol	C:38.17/37.56 H:4.80/4.78 N:13.91/13.43	7.84 / C ²
H-Glu(Gly ₅) Thia *TFA	C ₂₀ H ₃₀ N ₇ O ₁₀ SF ₃ 617.55 98 %	617.55 0.25/C 105-107°C	n.d. ¹	C:38.90/38.82 H:4.90/4.79 N:15.88/15.39	8.22 / C ²
H- Glu(PEG) Thia *HCl	92 %	≈8000 (mass emphasis)	n.d. ¹	n.d. ¹	n.d. ¹

¹ Not determined or not determinable.² HPLC separation conditions

Column: Nucleosil C-18, 7μ, 250 mm x 21 mm

Eluant: ACN/water/0.1% TFA

Gradient: 20 % ACN → 90 % ACN over 30 min; Flow rate: 6 ml/min; λ = 220 nm.

10

3.4 General synthesis procedures.

Method A: Peptide bond attachment by the mixed anhydride method using CFIBE as activation reagent.

15

10 mmol of N-terminally protected amino acid or peptide are dissolved in 20 ml of absolute THF. The solution is cooled to -15°C ± 2°C. With stirring in each case, 10 mmol of N-MM and 10 mmol of chloroformic acid isobutyl ester are added in succession, the stated temperature range being strictly adhered to. After approximately 6 min, 10 mmol of the amino component are added. When the amino component is a salt, a further 10 mmol of N-MM are then added to the reaction mixture. The reaction mixture is then stirred for 2 h in the cold state and overnight at room temperature.

20

The reaction mixture is concentrated using a rotary evaporator, taken up in EA, washed with 5% KH₂SO₄ solution, saturated NaHCO₃ solution and saturated NaCl solution and dried over NaSO₄. After removal of the solvent *in vacuo*, the compound is recrystallised from EA/pentane.

25

Method B: Peptide bond attachment by the mixed anhydride method using pivalic acid chloride as activation reagent.

30

5 10 mmol of N-terminally protected amino acid or peptide are dissolved in 20 ml of absolute THF. The solution is cooled to 0°C. With stirring in each case, 10 mmol of N-MM and 10 mmol of pivalic acid chloride are added in succession, the stated temperature range being strictly adhered to. After approximately 6 min, the mixture is cooled to -15°C and, once the lower temperature has been reached, 10
10 mmol of the amino component are added. When the amino component is a salt, a further 10 mmol of N-MM are then added to the reaction mixture. The reaction mixture is then stirred for 2 h in the cold state and overnight at room temperature. Further working up is carried out as in Method A.

Method C: Peptide bond attachment using TBTU as activation reagent.

15 10 mmol of the N-terminally protected amino acid or peptide and 10 mmol of the C-terminally protected amino component are dissolved in 20 ml of absolute DMF. The solution is cooled to 0°C. With stirring in each case, 10 mmol of DIPEA and 10 mmol of TBTU are added in succession. The reaction mixture is stirred for one
20 hour at 0°C and then overnight at room temperature. The DMF is completely removed *in vacuo* and the product is worked up as described in Method A.

Method D: Synthesis of an active ester (N-hydroxysuccinimide ester).

10 mmol of N-terminally protected amino acid or peptide and 10 mmol of N-hydroxy-succinimide are dissolved in 20 ml of absolute THF. The solution is
25 cooled to 0°C and 10 mmol of dicyclohexylcarbodiimide are added, with stirring. The reaction mixture is stirred for a further 2 h at 0°C and then overnight at room temperature. The resulting N,N'-dicyclohexylurea is filtered off, the solvent is removed *in vacuo* and the remaining product is recrystallised from EA/pentane.

Method E: Amide bond attachment using N-hydroxysuccinimide esters.

30 10 mmol of the C-terminally unprotected amino component are introduced into an NaHCO₃ solution (20 mmol in 20 ml of water). At room temperature and with stirring, 10 mmol of the N-terminally protected N-hydroxysuccinimide ester dissolved in 10 ml of dioxane are slowly added dropwise. Stirring of the reaction mixture is continued overnight and the solvent is then removed *in vacuo*.

35 Further working up is carried out as in Method A.

5 Method F: Cleavage of the Boc protecting group.

3 ml of 1.1N HCl/glacial acetic acid (*Method F1*) or 3 ml of 1.1N HCl/dioxane (*Method F2*) or 3 ml of 50 % TFA in DCM (*Method F3*) are added to 1 mmol of Boc-protected amino acid pyrrolidide, thiazolidide or peptide. The cleavage at RT is monitored by means of TLC. After the reaction is complete (approximately 2 h),
 10 the compound is precipitated out in the form of the hydrochloride using absolute diethyl ether, is isolated with suction and dried over P₄O₁₀ *in vacuo*. Using methanol/ether, the product is recrystallized or reprecipitated.

Method G: Hydrolysis.

1 mmol of peptide methyl ester is dissolved in 10 ml of acetone and 11 ml of 0.1M
 15 NaOH solution and stirred at RT. The course of the hydrolysis is monitored by means of TLC. After the reaction is complete, the acetone is removed *in vacuo*. The remaining aqueous solution is acidified, using concentrated KH₂SO₄ solution, until a pH of 2-3 is reached. The product is then extracted several times using EA; the combined ethyl acetate fractions are washed with saturated NaCl solution and
 20 dried over NaSO₄, and the solvent is removed *in vacuo*. Crystallization from EA/pentane is carried out.

5

Bibliography

- Ansorge, S., Schön, E., and Kunz, D. (1991). Membrane-bound peptidases of lymphocytes: functional implications. *Biomed.Biochim.Acta* 50, 799-807.
- 10 Ansorge, S., Bühling, F., Hoffmann, T., Kähne, T., Neubert, K., and Reinhold, D. (1995). *Di-peptidylpeptidase IV (CD26) in metabolism and the immune response* (Fleischer, B., Ed.) Springer-Verlag, Heidelberg. 163-184.
- 15 Augustyns, K., Bal, G., Thonus, G., Belyaev, A., Zhang, X. M., Bollaert, W., Lambeir, A. M., Durinx, C., Goossens, F., and Haemers, A. (1999). The unique properties of dipeptidyl-peptidase IV (DPP IV/CD26) and the therapeutic potential of DPP IV inhibitors. *Curr Medicinal Chem* 6, 311-327.
- 20 Buntrock, P., Neubert, K., Kohl, A., Moch, C., Born, I., Demuth, H.-U., and Barth, A. (1988). Stimulation and inhibition of wound healing process using short-chain peptides. *Biol.Zent.bl* 107, 87-92.
- 25 Demuth, H.-U., Heins, J., On the catalytic Mechanism of Dipeptidyl Peptidase IV. in *Dipeptidyl Peptidase IV*, Fleischer, Ed., R.G.Landes, Biomedical Publishers, Georgetown, 1, 1995.
- 30 Demuth, H.-U., McIntosh, C. H. S., Rosche, F., Pauly, R. P., Pederson, R. A., and Schmidt, J. Verfahren zur Senkung des Blutglukosespiegels in Säugern. DE 196 16 486.9
- 35 Fujita, K., Hagihara, M., Nagatsu, T., Iwata, H., and Miura, T. (1992). The activity of dipeptidyl peptidase II and dipeptidyl peptidase IV in mice immunized with type II collagen. *Bio-chem.Med.Metab.Biol.* 48, 227-234.
- 40 Gomez, S., Gluschkof, P., Lepage, A., and Cohen, P. (1988). Relationship between endo- and exopeptidases in a processing enzyme system: activation of an endoprotease by the ami-nopeptidase B-like activity in somatostatin-28 convertase. *Proc Natl Acad Sci USA* 85, 5468-5472.
- 45 Hegen, M., Niedobitek, G., Klein, C.E., Stein, H., and Fleischer, B. (1990). The T cell triggering molecule Tpl03 is associated with dipeptidyl aminopeptidase IV activity. *J.Immunol.* 144, 2908-2914.
- 50 Hegen, M., Mittrücker, H.-W., Hug, R., Demuth, H.U., Neubert, K., Barth, A., and Fleischer, B. (1993). Enzymatic activity of CD26 (dipeptidylpeptidase IV) is not required for its signalling function in T cells. *Immunobiol.* 189, 483-493.
- 50 Ishiura, S., Tsukahara, T., Tabira, T., and Sugita, H. (1989). Putative N-terminal splitting enzyme of amyloid A4 peptides is the multicatalytic proteinase,

- 5 ingensin, which is widely distributed in mammalian cells. *FEBS Lett* 257,
388-392.
- 10 Kameoka, J., Sato, T., Torimoto, Y., Sugita, K., Soiffer, R.J., Schlossman, S.F.,
Ritz, J., and Morimoto, C. (1995). Differential CD26-mediated activation of
the CD3 and CD2 pathways after CD6-depleted allogeneic bone marrow
transplantation. *Blood* 85, 1132-1137.
- 15 Kähne, T., Neubert, K., and Ansorge, S. (1995). Enzymatic activity of DPIV /
CD26 is involved in PMA-induced hyperphosphorylation of p56^{lck}
Immunol.Lett. 46, 189-193.
- 20 Kaspari, A., Diefenthal, T., Grosche, G., Schierhorn, A., Demuth, H.-U., Substrates
containing phosphorylated residues adjacent to proline decrease cleavage by
proline-specific pepti-dases, *Protein Struct Mol Enzym*, 147, 1996
- 25 Kessler, H. (1982). Konformation und biologische Wirkung von zyklischen
Peptiden. *Angew. Chem.* 94, 509
- 30 Kirschke, H., Barrett, A.J., and Rawlings, N.D. (1995). Proteinases 1: lysosomal
cysteine pro-teinas. *Protein Profile* 2, 1581-1643.
- 35 Kohl, A., Volk, H.D., Diezel, W., Buntrock, P., and Diamantstein, T. (1989). The
dipeptide Lys-Pro restores the diminished wound healing following
treatment with anti-T-helper cell monoclonal antibody.
Int.J.Immunopharmacol. 11, 237-240.
- 40 Kohl, A., Volk, H.D., Buntrock, P., Kohl, G., Diamantstein, T., and von Baehr, R.
(1991). The role of dipeptidylpeptidase IV positive T cells in wound healing
and angiogenesis. *Agents Actions* 32, 125-127.
- 45 Kräusslich, H.-G. and Wimmer, E. (1987). Viral Proteinases. *Ann. Rev. Biochem.*
57, 701
- 50 Kubota, T., Flentke, G.R., Bachovchin, W.W., and Stollar, B.D. (1992).
Involvement of dipep-tidyl peptidase IV in an in vivo immune response.
Clin.Exp.Immunol. 89, 192-197.
- Küllertz, G. and Boigk, J. (1986). Dipeptidyl peptidase IV activity in the serum and
synovia of patients with rheumatoid arthritis. *Z Rheumatol* 45, 52-56.
- Levy, M.T., McCaughan, G.W., Abbott, C.A., Park, J.E., Cunningham, A.M.,
Müller, E., Rettig, W.J., Gorell, M.D., Fibroblast Activation Protein: A Cell
Surface Dipeptidyl Peptidase and Gelatinase Expressed by Stellate Cells at
the Tissue Remodelling Interface in Human Cirrhosis *Hepatology*, 29,
1999, 1768-1778.

- 5 Nelson, P.J. and Krensky, A.M. (1998). Chemokines, lymphocytes and viruses:
what goes around, comes around. *Curr Opin Immunol* 10, 265-270.
- Niedermeyer, J., Enenkel, B., Park, J. E., Lenter, M., Rettig, W. J., Damm, K., and
Schnapp, A. (1998). Mouse fibroblast-activation protein - Conserved Fap
10 gene organization and bio-chemical function as a serine protease. *Eur J
Biochem* 254, 650-654.
- Novelli, M., Savoia, P., Fierro, M. T., Verrone, A., Quaglino, P., and Bernengo,
M.G. (1996). Keratinocytes express dipeptidyl-peptidase IV (CD26) in
15 benign and malignant skin diseases. *Br J Dermatol* 134, 1052-1056.
- Pauly, R. P., Demuth, H. U., Rosche, F., Schmidt, J., White, H. A., Lynn, F.,
McIntosh, C. H. S., and Pederson, R. A. (1999). Improved glucose tolerance
in rats treated with the dipeptidyl-peptidase IV (CD26) inhibitor ile-
20 thiazolidide. *Metabolism* 48, 385-389.
- Pauly, R. P., Rosche, F., Wermann, M., McIntosh, C. H. S., Pederson, R. A., and
Demuth, H. U. (1996). Investigation of glucose-dependent insulintropic
polypeptide-(1-42) and glucagon-like peptide-1-(7-36) degradation in vitro
25 by dipeptidyl-peptidase IV using matrix-assisted laser desorption/ionization
time of flight mass spectrometry - A novel kinetic approach. *J Biol Chem*
271, 23222-23229.
- Proost P., De Meester, I., Schols, D., Struyf, S., Lambeir, A. M., Wuyts, A.,
30 Opdenakker, G., De Clercq, E., Scharpe, S., and Van Damme, J. (1998a).
Amino-terminal truncation of chemokines by CD26/dipeptidyl-peptidase
IV. Conversion of RANTES into a potent inhibitor of monocyte chemotaxis
and HIV-1-infection. *J Biol Chem* 273, 7222-7227.
- 35 Proost, P., Struyf, S., Schols, D., Durinx, C., Wuyts, A., Lenaerts, J.P., De Clercq,
E., De Meester, I., and Van Damme, J. (1998b). Processing by
CD26/dipeptidyl-peptidase IV reduces the chemotactic and anti-HIV-1
activity of stromal-cell-derived factor-1alpha. *FEBS Lett* 432, 73-76.
- 40 Proost, P., Struyf, S., Schols, D., Opdenakker, G., Sozzani, S., Allavena, P.,
Mantovani, A., Augustyns, K., Bal, G., Haemers, A., Lambeir, A.M.,
Scharpe, S., Van Damme, J., and De Meester, I. (1999). Truncation of
macrophage-derived chemokine by CD26/dipeptidyl-peptidase IV beyond
its predicted cleavage site affects chemotactic activity and CC chemokine
45 receptor 4 interaction. *J Biol Chem* 274, 3988-3993.
- Raynaud, F., Bauvois, B., Gerbaud, P., and Evain Brion, D. (1992).
Characterization of specific proteases associated with the surface of human
50 skin fibroblasts, and their modulation in pathology. *J. Cell. Physiol.* 151,
378-385.

- 5 Reinhold, D., Bank, U., Buhling, F., Tager, M., Born, I., Faust, J., Neubert, K., and
Ansorge, S. (1997). Inhibitors of dipeptidyl peptidase IV (DP IV, CD26)
induces secretion of trans-forming growth factor-beta 1 (TGF-beta 1) in
stimulated mouse splenocytes and thy-mocytes. *Immunol Lett* 58, 29-35.
- 10 Reinhold, D., Hemmer, B., Gran, B., Born, I., Faust, J., Neubert, K., McFarland, H.
F., Martin, R., and Ansorge, S. (1998). Inhibitors of dipeptidyl peptidase
IV/CD26 suppress activa-tion of human MBP-specific CD4+T cell clones. *J*
Neuroimmunol 87, 203-209.
- 15 Reinhold D., Hemmer, B., Steinbrecher, A., Born, I., Faust, J., Neubert, K., Brocke,
S., Martin, R., Ansorge, S., Dipeptidyl Peptidase IV (CD 26): Role in T cell
activation and autoim-mune disease. *Biol. Chem*379, S 69, 1998.
- 20 Scholzen, T., Armstrong, C.A., Bunnett, N.W., Luger, T.A., Olerud, J.E., and
Ansel, J.C. (1998). Neuropeptides in the skin: interactions between the
neuroendocrine and the skin immune systems. *Exp Dermatol* 7, 81-96.
- 25 Shioda, T., Kato, H., Ohnishi, Y., Tashiro, K., Ikegawa, M., Nakayama, E.E., Hu,
H., Kato, A., Sakai, Y., Liu, H., Honjo, T., Nomoto, A., Iwamoto, A.,
Morimoto, C., and Nagai, Y. (1998). Anti-HIV-1 and chemotactic activities
of human stromal cell-derived factor 1alpha (SDF-1alpha) and SDF-1beta
are abolished by CD26/dipeptidyl peptidase IV-me-diated cleavage. *Proc*
Natl Acad Sci USA 95, 6331-6336.
- 30 Tanaka, T., Kameoka, J., Yaron, A., Schlossman, S.F., and Morimoto, C. (1993).
The costimu-latory activity of the CD26 antigen requires dipeptidyl
peptidase IV enzymatic activity. *Proc.Natl.Acad.Sci.U.S.A.* 90, 4586-4590.
- 35 Tanaka, S., Murakami, T., Horikawa, H., Sugiura, M., Kawashima, K., and Sugita,
T. (1997). Suppression of arthritis by the inhibitors of dipeptidyl peptidase
IV. *Int J Immunophar-macol* 19, 15-24.
- 40 Tanaka, S., Murakami, T., Nonaka, N., Ohnuki, T., Yamada, M., and Sugita, T.
(1998). Anti-arthritic effects of the novel dipeptidyl peptidase IV inhibitors
TMC-2A and TSL-225. *Immunopharmacology* 40, 21-26.
- 45 Vanhoof, G., Goossens, F., De Meester I, Hendriks, D., and Scharpe, S. (1995).
Proline motifs in peptides and their biological processing. *FASEB J.* 9, 736-
744.
- Wallengren, J. (1997). Vasoactive peptides in the skin. *J Investig Dermatol Symp*
Proc 2, 49-55.
- 50 Yaron, A. and Naider, F. (1993). Proline-dependent structural and biological
properties of peptides and proteins. *Crit.Rev.Biochem.Mol.Biol.* 28(1), 31-
38.